Biological Sciences Section

Pakistan J. Sci. Ind. Res., Vol. 13, No. 3, October 1970

STUDIES IN THE BIOCHEMISTRY OF MICROORGANISMS

Part VIII*.—Isolation and Characterization of Penicillium martinsii Biourge Metabolic Products. The Structure of Amudol

AHMAD KAMAL† and CHARLES H. JARBOE

Medicinal Chemistry Section, Department of Pharmacology, School of Medicine, University of Louisville, Louisville, Kentucky, U.S.A.

IZHAR H. QURESHY, (Miss) SHAHEEN A. HUSAIN, (Mrs.) NAJMA MURTAZA and (Miss) RADIA NOORANI

P.C.S.I.R. Laboratories, Karachi 39

ASAF ALI QURESHI

Department of Organic Chemistry, Yale University, New Haven, Connecticut, U.S.A.

(Received November 28, 1969)

Penicillium martinsii Biourge, grown on a semisynthetic medium, produces five crystalline compounds, viz., *amudol*, $C_7H_7O_3Cl$, m.p. 146–47°C; amudane, m.p. 219–20°C; amudene, m.p. 270–72°C; ergosterol, m.p. 165–57°C; and mannitol.

On the basis of chemical and spectral evidence amudol has been shown to be the hitherto undescribed 2,5-dihydroxy-4-chlorobenzyl alcohol. Amudol has been shown to have antibacterial properties.

Penicillium martinsii was first described by Biourge[1] in 1930. However, no attempt was made to investigate its metabolic products for well over two decades. In 1956, Wirth, Gilmore and Naval[2] published a report claiming to have isolated the antibiotic, penicillic acid from this mold. The reported ability of *Penicillium martinsii* Biourge, to produce antibiotic substances evinced interest in us to make a systematic investigation of its metabolic products. This report concerns the isolation, identification and antibacterial effects of amudol, one of the metabolic products of the mold.

The mold was first grown on a semisynthetic medium: Czapek–Dox-enriched with carrot extract. Ethyl acetate extraction of the culture filtrate gave an oily product with ν_{max} at 3333 and 1720 cm^{-1}. This oily ketol, however, could not be crystallized. Similar results were obtained using the medium developed by Moyer[3] which contained corn starch in place of corn steep liquor. However, when *Penicillium martinsii* Biourge, was grown on Moyer's medium (*loc. cit.*) enriched with carrot extract, an extraction of the broth with ethyl ether gave a crystalline product, hereafter termed amudol, m.p. 146–47° (1.93 g from 4.2 l.). Slightly better yields (2.1 g from 4.2 l.) were obtained when molasses was used in the above medium in place of lactose.

In alcohol amudol gives a transient bluish green color with alcoholic ferric chloride. It gives a positive Beilstein test for chlorine and is optically inactive. Its mass spectrum shows a molecular ion peak at m/e 174 and its elemental analysis agrees with the molecular formula $C_7H_7O_3Cl$.

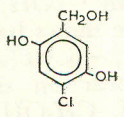
A band at 1590 cm^{-1} in the infrared is indicative of a benzene ring which accounts for all unsaturation. A band at 3420 cm^{-1} (broad) indicates the presence of OH. The PMR spectrum of amudol in deuteroacetone shows a singlet corresponding to two phenolic hydroxyls at τ 2.17. At τ 6.70 there is a singlet corresponding to one

*Part VII. Pakistan J. Sci. Ind. Res., 11, 367 (1968).

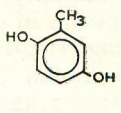
† To whom communications should be sent at the following location: P.C.S.I.R. Laboratories, Country Club Road, Karachi 32, Pakistan.

alcoholic hydroxyl. Both these bands disappear on shaking with D_2O. A singlet occurring at τ 5.32 indicates CH_2—OH next to a benzene ring. The compound shows a two benzenoid proton singlet at τ 3.31. This singlet became a doublet with a small coupling constant (J=0.1 c/s) when the spectrum was run on the Perkin-Elmer HA100 instrument, indicating the protons to be *para*.

On the basis of the above spectral evidence, amudol, has been assigned the structure: 2,5-dihydroxy-4-chlorobenzyl alcohol (I). Compelling evidence in support of this structure was deduced from its transformation into 2,5-dihydroxytoluene (II) on catalytic hydrogenolysis.



I



II

The fragmentation pattern shown by the mass spectrum of amudol is also consistent with this structure It shows a molecular ion peak at m/e 174 corresponding to the molecular formula $C_7H_7O_3Cl^+$. A second intense peak (base peak) appears at m/e 156 (m* 140) and is due to the loss of one molecule of water. This ion undergoes fragmentation to give ions at m/e 128 (m* 105) and 121 (m* 93.9) derived from the loss of chlorine and carbon monoxide. It also shows loss of hydroxyl group (M^+–17; m/e 157) from the molecular ion. Other prominent peaks are at m/e 129, 110, 93, 76, 75, 67 and 51. The general breakdown sequence is outlined in Chart I. The majority of transitions indicated are supported by appropriate metastable peaks.

As indicated in Table 1 amudol shows antibiotic activity against *Bacillus subtilis*, *B. anthracis*, *Staphylococcus albus*, *Sarcina lutea*, *Salmonella typhosa*, *S. paratyphi A*, *S. paratyphi B* and *Escherichia coli*. No other crystalline material was obtained from the broth.

The mold mycellium was sequentially extracted with petroleum ether (60–80°), ethyl ether, ethyl acetate, chloroform and ethanol in a soxhlet extractor. The petroleum ether extract afforded a crystalline material, m.p. 165–°C. This gave a positive Liebermann–Burchard test for steroids. It was found to be ergosterol.

The ethyl ether, ethanol and chloroform extracts contained a crystalline material, m.p. 244–45°. Since the yield of this material was

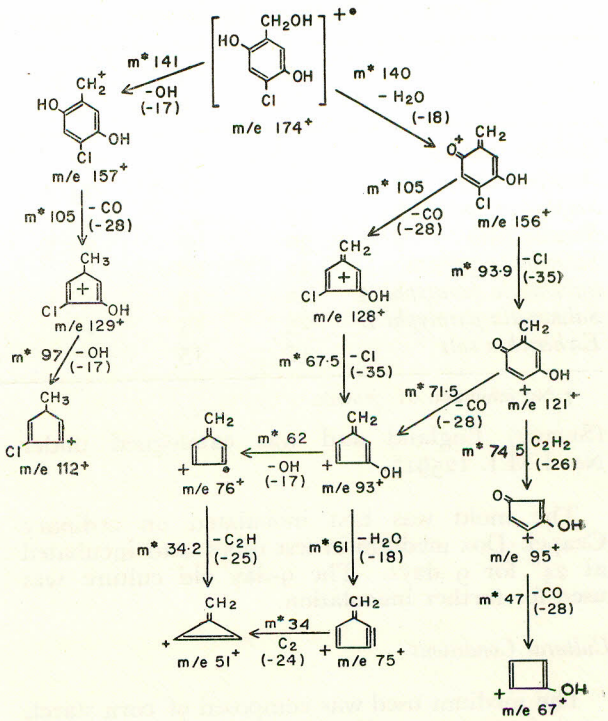


Chart I.—Fragmentation pattern of amudol.

maximum in chloroform, that solvent was used to recover this second product subsequent to petroleum ether extraction of the steroid fraction. Careful fractional crystalization of the chloroform extractable product gave two pure compounds: amudane, m.p. 219–20° (obtained in minute quantities), and amudene, m.p. 270–72°. Further work on these two products is proceeding and will be reported later.

Experimental

All m.ps are corrected. UV spectra were determined with a Beckman spectrometer Model DK-2 and methanol was used as solvent. IR spectra were determined with a Beckman IR-5. PMR spectra were recorded on Varian A60 and Perkin-Elmer HA100 instruments using tetramethylsilane as an internal reference. Mass spectra were determined on a Perkin-Elmer Model RMU-6 spectrometer.

Organism

During studies of *Penicillium* species from West Pakistan, one of us (N.M.) reported the isolation of *Penicillium martinsii* Biourge, from the soil of Karachi,[4] which was confirmed by the Commonwealth Mycological Institute, Kew

TABLE 1.—ZONES OF INHIBITION (IN mm) FOR DIFFERENT CONCENTRATIONS OF AMUDOL

	2 %	1 %	0.5 %	0.25 %	0.125 %	0.062 %	0.031 %	0.015 %	Control
Bacillus subtilis	27*	25	24	22	21.7	21	20	+	+
Bacillus anthracis	29	25.5	24.5	21.5	20	19	18	+	+
Staphylococcus albus	47	45	43	35.5	29.5	27	25	+	+
Sarcina lutea	30	29	26.5	23.5	22.5	21	19.5	+	+
Salmonella typhosa	31	28	18	16	15	+	+	+	+
Salmonella paratyphi A	26	19	18	15	+	+	+	+	+
Salmonella paratyphi B	25	25	17	+	+	+	+	+	+
Escherichia coli	17	15	+	+	+	+	+	+	+

† No inhibition of growth.

(Surrey) England and was catalogued under No. I.M.I. 125917.

The mold was first inoculated on ordinary Czapek–Dox medium in test tubes and incubated at 24° for 9 days. The 9-day old culture was used for further inoculation.

Cultural Conditions

The medium used was composed of corn starch 20 g, lactose 44 g, $NaNO_3$ 3 g, KH_2PO_4 9.5 g, $MgSO_4.7H_2O$ 0.25 g, $ZnSO_4$ 0.44 g, glucose monohydrate 2.75 g and carrot extract, 1 l. (prepared according to the method of Kamal and his coworkers[5].) In some experiments molasses (120g) was used in place of lactose in the above medium.

In a typical batch, twelve (1-l.) flasks, each containing 350 ml of the above medium (pH 5) were inoculated with 9-day old tube cultures of *Penicillium martinsii* Biourge, and incubated at 24°. After 17 days the mycellium was removed by filtration (suction). The broth and mycellium were extracted separately.

Broth Isolation of Amudol

The broth (2.7 l.), pH 6, was extracted with ether. The organic layer was dried (anhydrous Na_2SO_4) and the solvent removed to give a brown semisolid (2.52 g from 3.2 l. metabolic solution). Crystallization from ethyl acetate–petroleum ether and recrystallization from ethyl ether gave light brown needles (1.93 g), m.p. 146–47°. In solution the color slowly darkens on heating or exposure to air. The compound gives a transient blue color with alcoholic ferric chloride. (Found: C, 48.47; H, 4.28; O, 26.78; Cl (by difference) 20.47. Calc. for $C_7H_7O_3$ Cl: C, 48.13; H, 4.01; O, 27.50; Cl, 20.34%.)

Amudol shows λ_{max} at 298 mμ (log ε 3.37). The IR spectrum shows bands (KBr) at 1590 cm^{-1} (aromaticity) and 3420 cm^{-1} (OH). Its PMR spectrum in deuteroacetone shows bands at τ 2.17 (singlet; 2-O*H*, phenolic); τ 6.70 (singlet; 1-O*H*, alcoholic) which disappear on shaking with D_2O; τ 7.32 (singlet; 2-H, C*H*$_2$OH) and τ 3.31 (singlet; 2-aromatic H). The last band showed a doublet with a small coupling constant (J=0.1 c/s) on Perkin-Elmer HA100 instrument.

Mycellium

The mycellium was dried at 60° (204 g of dried material from 12 flasks). In a typical experiment the dried mycellium (100 g) was continuously extracted in a soxhlet extractor using (a) petroleum ether (60–80°) (2 days); (b) chloroform (2 days) and (c) ethanol (4 days).

(a) *Petroleum Ether* (60–80°) *Extract.*—After extraction with petroleum ether (2 days), the extract on removal of solvent gave a colorless product which crystallized from ethyl ether to yield colorless needles, m.p. 160° (0.51 g), $[\alpha]_D^{27}$ −128. It gave positive Liebermann–Burchard test for sterols and was identified as ergosterol from its mixed m.p. (undepressed) and identical UV and IR absorption spectra.

(b) *Chloroform Extract.*—After extraction with petroleum ether (60–80°) the mycellium was extracted with chloroform (2 days). On removal of the solvent the extract gave a colorless solid m.p. 244–45° (1.67 g) which gave a positive Beilstein test for chlorine. Repeated fractional tion from ether–benzene and benzene–ethyl acetate crystallizamixtures gave two products: amudane m.p. 119–20°, and amudene m.p. 270–72°.

(c) *Ethanol Extract.*—The mycellium from above (b) when extracted with ethanol (4 days) gave a crystalline material which on crystallization from the same solvent was obtained pure as colorless needles, m.p. 164–65° (0.6 g). The compound gave a positive Molish test for carbohydrates.

It was identified as mannitol by mixed m.p. with an authentic sample. It gave a hexaacetate, m.p. 119–20°, identical with mannitol hexaacetate.

Catalytic Reduction of Amudol

Amudol (0.87 g; 0.005 mole) was taken up in methanol (50 ml) and reduced with hydrogen using 5% Pd–charcoal catalyst (0.25 g). When hydrogen absorption ceased (2 hr; 225 ml) the catalyst was removed by vacuum filtration through celite bed. Removal of the solvent gave a crystalline residue which was purified by crystallization from a benzene–ether mixture to give colorless plates: m.p. 124° (undepressed on mixture with an authentic sample of 2,5-dihydroxytoluene). Thin layer chromatography on Merck's silica-gel G (solvent: ether–benzene, 9:1) gave R_f 0.7, the same as that of an authentic sample. (Found: C, 67.87; H, 6.40; O, 25.60. Calc. for $C_7H_8O_2$; C, 67.73; H, 6.50; and O, 25.7%.) UV absorption bands appear at λ_{max} 292 mμ (log ε 3.44) with a shoulder at 226 mμ (log ε 3.59). Its PMR spectrum determined on a Varian A60 instrument in deuteroacetone shows resonances corresponding in chemical shifts and intensity to the groupings: ArCH_3 (singlet; τ 8.32), ArOH (doublet; τ 2.89; J=6.5 c/s) which disappear with D_2O and ArH (multiplet; τ 3.80; J=8 c/s for *o*-coupling; J=2 c/s for *m*-coupling and J=0.2 c/s for *p*-coupling.

Acknowledgment.—Thanks are due to Professor A.I. Scott, Yale University, for his help in providing us the sample of 2,5-dihydroxytoluene for comparison. Thanks are also due to the National Science Foundation for the grant of a Senior Scientist Fellowship to one of us (A.K.) and to the University of Louisville for sponsorship of the grant.

References

1. K.B. Raper and C. Thom, *Manual of Penicillia* (1949), pp. 500–503.
2. J.C. Wirth, T.E. Gilmore and J. J. Naval, Arch. Biochem. Biophys., **63,** 452 (1956).
3. A. J. Moyer and R.D. Coghill, J. Bact., **53,** 57 (1946); *cf.* Ref. 1, p. 92.
4. Syed Iftikhar Ahmad and Najma Murtaza, unpublished work.
5. A. Kamal, Asaf A. Qureshi and M. Ali Khan, Tetrahedron, **19,** 117 (1963).